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# Differential effects of peroxisome proliferator activated receptor-γ (PPARγ) ligands in proximal tubular cells: Thiazolidinediones are partial PPARγ agonists

**RAVINDER S. CHANA, ANDREW J. LEWINGTON, and NIGEL J. BRUNSKILL**

*Department of Cell Physiology and Pharmacology, and Department of Nephrology, University of Leicester, Faculty of Medicine and Biological Sciences, Leicester, England, United Kingdom*

**Differential effects of peroxisome proliferator activated receptor-γ (PPARγ) ligands in proximal tubular cells: Thiazolidinediones are partial PPARγ agonists.**

*Background.* Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors with multiple effects on target cell function. PPARγ activity is regulated by extracellular signal-regulated protein kinase (ERK), mitogen-activated protein (MAP) kinase, and PPARγ ligands have varying effects on activity of ERK. Different PPARγ ligands have been shown to have both protective and detrimental effects in the kidney. Since transcriptional activation by different PPAR agonists is ligand- and depot-specific PPARγ, we have examined the effects of different agonists on PPAR activity in the proximal tubule.

*Methods.* Opossum kidney cells were used in all experiments, transiently transfected with a PPAR response element luciferase reporter and subject to stimulation with various PPAR ligands. The role of ERK and phosphorylation in PPARγ activation were studied, as were the effects of PPAR agonists on ERK activation and cell proliferation.

*Results.* Transcriptional activity of PPAR was not stimulated by PPARα agonists, and only very modestly stimulated by a PPARβ agonist. The PPARγ agonists 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$), ciglitazone, and troglitazone stimulated significant transcriptional activation and phosphorylation of PPARγ. These effects were more marked with 15d-$PGJ_2$. Thiazolidinediones attenuated 15d-$PGJ_2$ evoked PPARγ activation and phosphorylation. ERK activity positively regulated PPAR activation. Only 15d-$PGJ_2$ stimulated ERK activity and cell proliferation, and these effects were also inhibited by thiazolidinediones.

*Conclusion.* PPARγ agonists exert differential effects in proximal tubule cells with thiazolidinediones behaving as partial agonists.

**Key words:** proximal tubule, PPAR, thiazolidinedione, ciglitazone, troglitazone prostaglandin, partial agonist, MAP kinase, phosphorylation.

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The peroxisome proliferator activated receptors (PPARs), PPARα, -β/δ, and -γ, are ligand-activated transcription factors belonging to the nuclear hormone superfamily that also includes the retinoic acid and thyroid hormone receptors. Upon ligand binding, PPARs form heterodimers with one of the three retinoid X receptor proteins which then bind to PPAR response elements (PPRE) within the promoter regions of target genes. They have been shown to regulate diverse cell functions, including adipocyte differentiation, control of inflammation, fatty acid metabolism, cell cycle control, and the development of atherosclerosis [1–3]. A variety of endogenous and exogenous ligands for PPARs have been identified [4]. PPARα is the target for the hypolipidemic fibrate drugs, and PPARγ the target for the antidiabetic thiazolidinediones. Most fatty acids can also activate PPARs. A number of eicosanoids have also been shown to activate PPARs (e.g., 5, 8, 11, 14 eicosatetraynoic acid (ETYA) for PPARα, and 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) for PPARγ.

Although all three PPAR subtypes have been identified in mammalian kidneys [5–7], their role in regulating renal (patho)physiology is only just beginning to be unraveled. The most widely investigated subtype in the context of kidney function is PPARγ, and some animal studies have suggested a protective effect of thiazolidinediones in both diabetic and nondiabetic models of renal disease [8, 9]. However, the response of glomerular cells to PPARγ activation may be agonist dependent, with some agonists potentially augmenting detrimental inflammatory responses in glomerulonephritis [10]. In vitro, PPARγ agonists suppress secretion of type I collagen by mesangial cells [11] and may protect mesangial cells from lipid-induced injury [12]. In contrast, stimulation of PPARγ by fatty acids presented to proximal tubular cells bound to albumin results in profound apoptosis [13].

Transcriptional activation by different PPAR agonists is ligand- and depot-specific. Different thiazolidinediones

acting through PPARγ may induce differential gene transcription, and the effects of PPARγ agonists are likely to differ between cell types [14–18]. Thus the effects of PPAR activation in kidney cells are likely to be complex and context specific, and it remains unclear under what circumstances PPAR activation in the kidney may be beneficial or deleterious.

Mitogen-activated protein (MAP) kinases are key mediators involved in the intracellular network of interacting proteins that transduce extracellular stimuli to intracellular responses. Extracellular signal-regulated kinases (ERK) were the first reported and are the best described members of the MAP kinases. The two ERK isoforms, ERK1 (or p44 MAP kinase) and ERK2 (or p42 MAP kinase), are serine/threonine kinases that regulate the expression of many genes via the phosphorylation of numerous families of transcription factors [19, 20]. Activity of ERK has been shown to have a central role in the development of both glomerular and tubular dysfunction [21, 22, 23].

PPARγ is a phosphoprotein whose activity is regulated by phosphorylation in addition to ligand binding. The MAP kinases, particularly ERK, may mediate this phosphorylation, but data regarding the effects of ERK-induced PPARγ phosphorylation on PPARγ activation are conflicting. Hu et al [24] reported that ERK mediated phosphorylation of PPARγ resulted in reduced transcriptional activity and inhibition of adipocyte differentiation. Conversely Zhang et al [25] demonstrated the opposite effect of ERK on PPARγ transcriptional activity in transiently transfected Chinese hamster ovary (CHO) cells [25]. Subsequently, the balance of evidence has supported an inhibitory role for ERK in the regulation of PPARγ transcriptional activity [26–28]. Similar tension exists in the literature regarding the ability of PPARγ ligands to activate ERK. In mesangial cells 15d-$PGJ_2$, but not the thiazolidinedione ciglitazone, is able to activate ERK independently of PPARγ [29]. Human T cells have also been shown to respond in a similar manner to 15d-$PGJ_2$ [30], whereas in astrocytes and preadipocytes ERK is activated by both 15d-$PGJ_2$ and ciglitazone [31].

Although PPAR agonists have shown promise in the treatment of kidney disease, their role in the modulation of renal pathology requires further clarification. In particular studies of progression of nephropathy in diabetic patients treated with thiazoidinediones are not yet available, but will be of considerable interest given the observed pleiotropic effects of these agents on renal cell function. An intriguing question is whether a PPARγ agonist with beneficial effects on proximal tubular cell function, such as a thiazolidinedione, may be used to antagonize the potentially deleterious effects of other PPARγ agonists such as albumin bound fatty acids [13]. We have therefore examined the effects of various PPAR ligands to activate PPAR in opossum kidney proximal tubular cells, and particularly studied the differences and similarities in signaling between the thiazolidinediones and 15d-$PGJ_2$ in these cells.

## METHODS

### Reagents and cell culture

Opossum kidney cells were originally obtained from J Caverzasio and grown in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) mix supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin under standard culture conditions.

General laboratory chemicals were obtained from Sigma (Poole, UK) and were of the highest grade available. The PPAR agonists 15d-$PGJ_2$, 5, 8, 11, 14-eicosatetraynoic acid (ETYA), 4-chloro-6-(2,3-xylindo)-2-pyrimidinylthioacetic acid (WY-14643), ciglitazone, and troglitazone were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). The PPARβ agonist carbaprostacyclin was obtained from Alexis Corporation (Nottingham, England). 9 cis retinoic acid was obtained from Sigma as were all general laboratory chemicals that were of the highest grade available. [$^{32}$P]-orthophosphate was obtained from Amersham (Amersham Pharmacia Biotech, Little Chalfont, England).

The constitutively active MAP kinase kinase (MEK1) mutant expressing plasmid, pFC-MEK1 (pMEK1-CA), was obtained from Stratagene (La Jolla, CA, USA), the kinase dead mutant of MEK1 in pCMV5 (pMEK1-KD) was provided by Dr Jonathan Blank (Leicester, England). The reporter plasmid pPPRE-TK-Luc was kindly provided by Dr M. Lazar (Philadelphia, PA, USA). Fugene 6 transfection reagent was purchased from Roche Diagnostics (Lewes, England). Luciferase assays were performed using LucLite kit (Packard, Pangbourne, England). β-galactosidase assay kits were obtained from Promega (Madison, WI, USA). Antibodies against active phosphorylated ERK (pERK) and total ERK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and supplied by Insight Biotechnology (London, England). Antimouse horseradish peroxidase (HRP)-conjugated secondary antibody and antirabbit HRP-conjugated secondary antibody were purchased from Sigma.

### Transient transfection and luciferase reporter assays

Opossum kidney cells were transfected with pPPRE-TK-Luc, pMEK1-CA, pMEK1-KD, and pSVβ-Gal using Fugene-6 according to the manufacturer's instructions. Briefly, 25,000 cells per well plated in 24-well plates were grown overnight to approximately 50% confluency. Cells were then transfected with various combinations of pPPRE-TK-luc, pMEK1-CA, and pMEK1-KD, together with pSVβgal to control for transfection efficiency,

in complete DMEM/F-12 medium for 24 hours. During transfection the DNA ratio was kept constant in all experiments such that 0.25 μg of each plasmid DNA was used per well and the quantity of Fugene-6 varied according to the quantity of plasmid DNA.

After transfection, the cells were growth arrested in serum-free DMEM/F-12 medium for 24 hours. Cells were then stimulated in this medium containing the PPAR agonists 15d-$PGJ_2$ (0 to 20 μmol/L), ciglitazone or troglitazone (0 to 20 μmol/L), ETYA (0 to 20 μmol/L), WY14643 (0 to 20 μmol/L), carbaprostacyclin (0 to 20 μmol/L), 9-cis retinoic acid (0 to 20 μmol/L), and vehicle [either 0.1% dimethyl sulfoxide (DMSO), or 0.1% ethanol]. After 24 hours, the media was removed and cells lysed in a buffer consisting of 500 mmol/L HEPES, 1 mmol/L $CaCl_2$, 1 mmol/L $MgC_2$ containing 2% Triton N101, pH 7.8. Cells were lysed for 10 minutes, 100 μL aliquots of lysate were assayed for luciferase, the remainder being used for β-galactosidase assay. Luciferase activity was measured using a Lumicount luminometer (Packard, Pangbourne, England) and β-galactosidase using a Spectrocount spectrophotometer (Packard).

### Assessment of cell viability and proliferation

Cell viablilty in response to PPAR and other agonists was determined by methylthiazoletetrazolium (MTT) assay. Opossum kidney cells, prestimulated for 24 hours with varying concentrations of agonists, were incubated with 50 μL of 2 mg/mL MTT solution for 1 hour and solubilized in DMSO. The formation of the formazan product was measured with a spectrophotometer set to record absorbance at 540 nm.

Cell proliferation was assessed [$^3$H] thymidine assay as previously described [32]. Briefly, cells, growing in 96-well plates, were exposed to varying concentrations of PPAR agonists. Incorporation of [$^3$H] thymidine (3.6 μCi/mL culture medium) into nuclear DNA was terminated after 3 hours by washing with phosphate-buffered saline (PBS). The cells were fixed at 4°C by first adding 50% methanol/10% glacial acetic acid for 30 minutes and then trichloroacetic acid (10%) for 10 minutes, before being solubilized overnight in 100 mmol/l sodium hydroxide solution containing 1% sodium dodecyl sulfate (SDS). Radioactivity was counted using a beta scintillation counter.

### [$^{32}$P]-orthophosphate metabolic labeling of cells and immunoprecipitation with anti-PPARγ antibody

Opossum kidney cells were metabolically labeled with [$^{32}$P]-orthophosphate as previously described [32]. Briefly, cells grown to confluence in 6-well plates were growth arrested in serum-free medium overnight, washed, and then treated with serum-free, phosphate-free DMEM/F-12 medium containing either 200 μCi/mL [$^{32}$P]-orthophosphate alone or with this medium containing $PGJ_2$ (5 μmol/L) and ciglitazone (5 μmol/L) for 4 hours at 37°C. After stimulation, the cells were washed twice with ice cold PBS, pH 7.4, and lysed in lysis buffer consisting of 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 200 μmol/L phenylmethylsulfonylfluoride (PMSF), and 200 μmol/L sodium vanadate prepared in PBS, pH 7.4. Lysates were collected after 20 minutes incubation and then precleared with the addition of protein A-sepharose for 30 minutes at 4°C. The supernatants were then incubated overnight with 10 μL aliquots of anti-PPARγ antibody at 4°C. The resulting immune complexes were immunoprecipitated with protein A-sepharose, washed three times with cold lysis buffer and prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by the addition of 30 μL of Laemmli buffer containing 60 mmol/L Tris, pH 6.8, 10% glycerol, 2% SDS, 100 mmol/L dithiothreitol (DTT), and 0.01% bromophenol blue. The immunoprecipitated proteins were subjected to 10% SDS-PAGE and radiolabeled proteins detected by autoradiography. For densitometric analysis, bands on exposed x ray film derived from immunoprecipitation experiments were scanned and quantified using Scion Image version 4.0.2.

### Assessment of MAP kinase activation

The MAP kinase activation in opossum kidney cells in response to PPARγ agonists was examined by Western blot analysis. Cells grown to 50% confluency in 24-well plates were growth arrested in serum-free medium for 24 hours and then treated with $PGJ_2$ (5 μmol/L), ciglitazone (5 μmol/L), troglitazone (5 μmol/L), or medium alone containing vehicle. After incubation at varying time points, cells were washed and lysed in Laemmli buffer as described above. After PAGE and Western blotting of lysate proteins, pERK was detected using a monoclonal anti-pERK antibody (1:1000) and secondary HPR-linked antimouse IgG antibody (1:5000). Total ERK was detected with polyclonal anti-ERK antibody (1:1000) and secondary HRP-linked antirabbit IgG antibody (1:5000). Bound antibodies were visualized using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

## RESULTS

Our first aim was to establish a repertoire of responses in opossum kidney cells to stimulation with a variety of potential agents likely to influence PPAR activation via PPRE (Fig. 1). Treatment of opossum kidney cells with the PPARα ligands WY14643 and ETYA caused no significant stimulation of PPRE-driven luciferase expression. Conversely, treatment with the PPARγ ligands 15-d$PGJ_2$ and the thiazolidinediones ciglitazone and troglitazone stimulated significant luciferase expression (187.9% ± 34%, 137.1% ± 10%, 133.9% ± 11.6% of

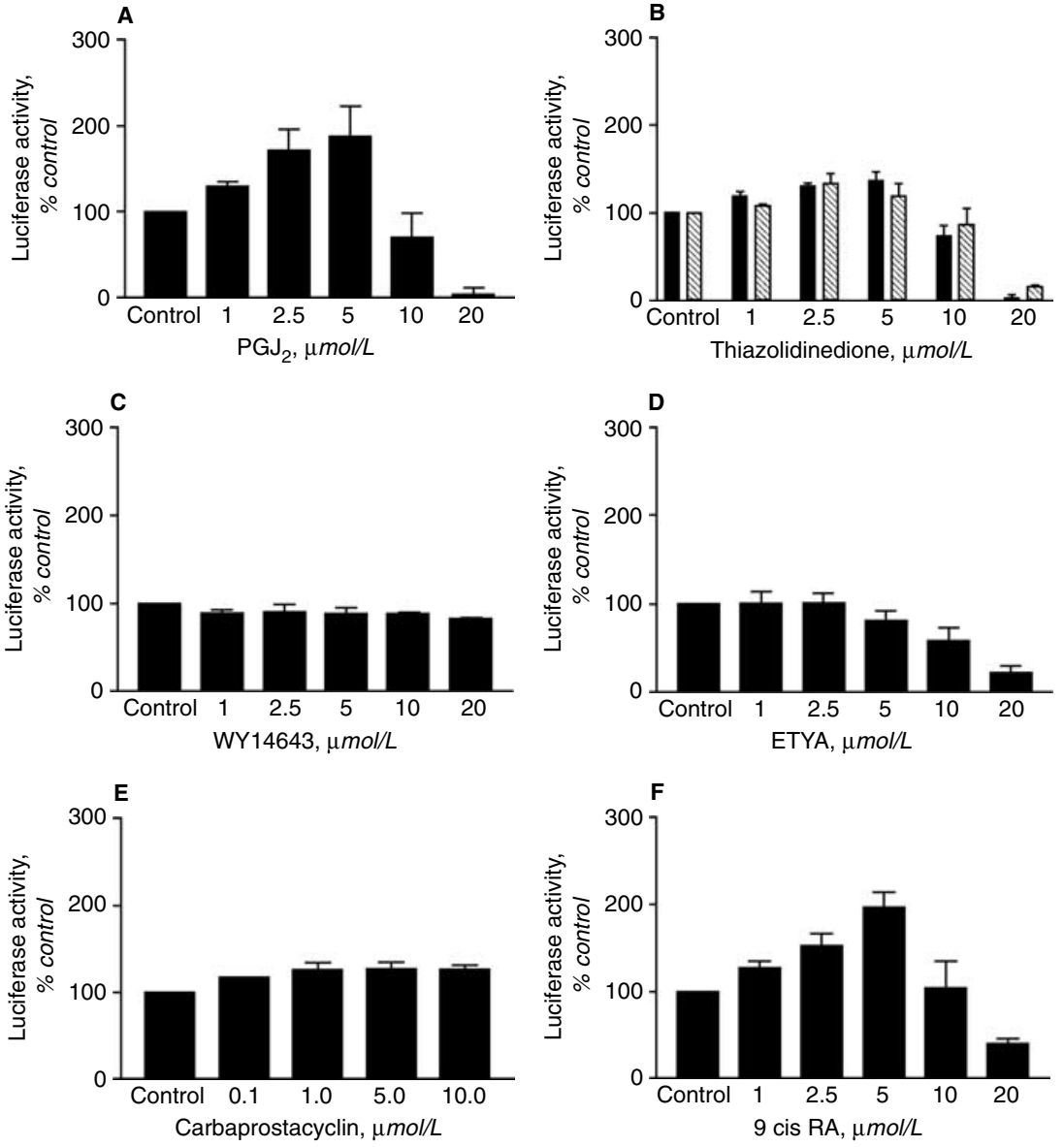


**Fig. 1. Activation of peroxisome proliferator activated receptors (PPAR) response elements (PPRE) by various PPAR ligands in opossum kidney cells.** Opossum kidney cells were transiently transfected with pPPRE-TK-luc and then stimulated with increasing concentrations of PPARγ ligands 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) (*A*) and ciglitazone (■) or troglitazone (▨) (*B*), PPARα ligands WY14643 (*C*), 5, 8, 11, 14 eicosatetraynoic acid (ETYA) (*D*), PPARβ ligand carbaprostacyclin (*E*), and retinoic acid receptor ligand 9-cisRA (*F*). All luciferase values are given as a percentage of nonstimulated controls. Values are expressed as means ± SEM. Each condition was measured in triplicate in three to six experiments.

control, respectively, at 5 μmol/L). The PPARβ agonist carbaprostacyclin induced a very modest increase in luciferase activity (125% ± 7.2% of control at 5 μmol/L), whereas the ligand of the retinoic acid receptor 9-cis retinoic acid elicited a robust stimulation of luciferase activity (197.2% ± 16.7% of control at 5 μmol/L).

We went on to examine the effect on PPRE-driven luciferase expression of combining different PPARγ agonists and/or 9-cis retinoic acid together (Fig. 2). The combination of 15d-$PGJ_2$ and 9-cis retinoic acid resulted in significant augmentation of PPRE activation over and above that observed with either agent alone. Conversely, when 9-cis retinoic acid was combined with ciglitazone, no enhancement was observed; indeed the level of luciferase expression was lower than that expected in the setting of 9-cis retinoic acid stimulation alone (Fig. 2A). Furthermore, when pPPRE transfected cells were treated with 5 μmol/L 15d-$PGJ_2$ combined with varying concentrations of ciglitazone or troglitazone (Fig. 2B), the luciferase expression expected with 5 μmol/L 15d-$PGJ_2$ alone was reduced in a dose-dependent manner.

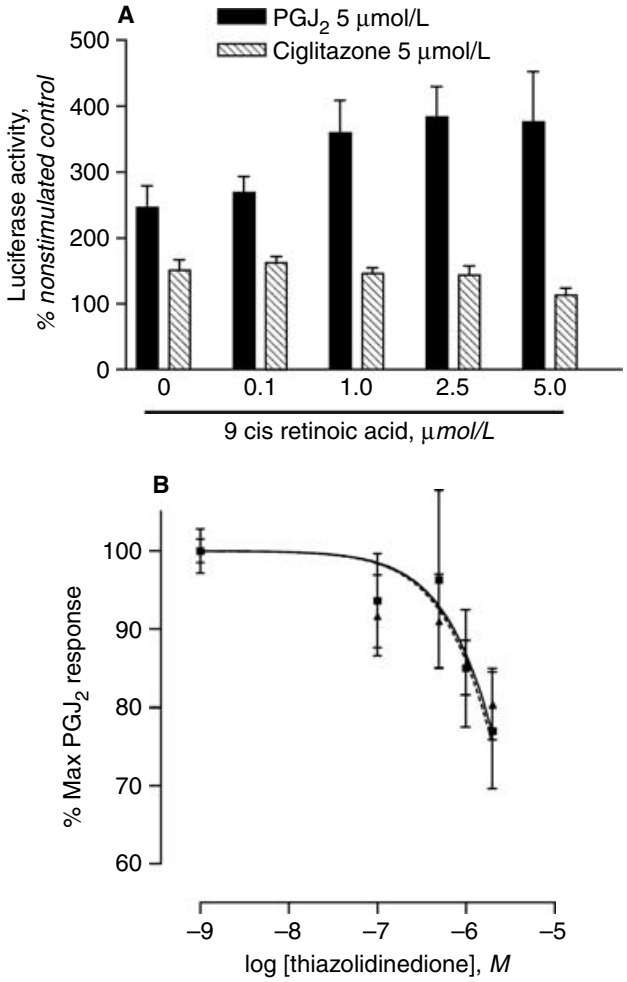


**Fig. 2. Effect on peroxisome proliferator activated receptors (PPAR) response elements (PPRE) activation in opossum kidney cells of combining PPAR and retinoic acid receptor ligands.** (*A*) Opossum kidney cells were transiently transfected with pPPRE-TK-luc and then stimulated with either 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$)or ciglitazone alone and with varying concentrations of retinoic acid. Luciferase values are given as a percentage of nonstimulated controls. (*B*) Opossum kidney cells were transiently transfected with pPPRE-TK-luc and then stimulated with 5 μmol/L 15d-$PGJ_2$ and varying concentrations of ciglitazone (■, unbroken line) or troglitazone (▲, broken line). Luciferase values are given as a percentage of the maximal response seen with 5 μmol/L 15d-$PGJ_2$ alone. Values are expressed as means ± SEM. Each condition was measured in triplicate in four experiments.

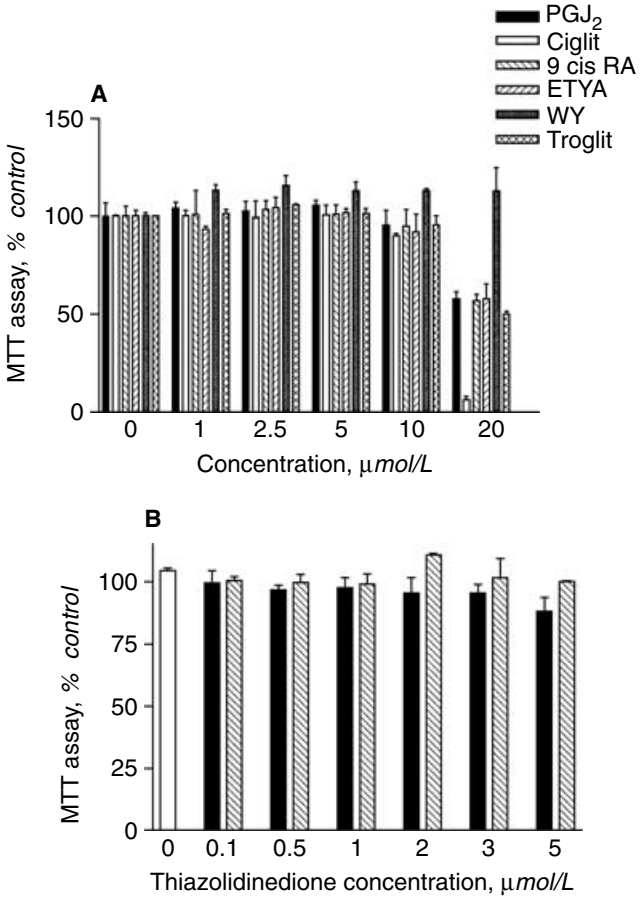


**Fig. 3. Toxicity of peroxisome proliferator activated receptors (PPAR) ligands in opossum kidney cells.** (*A*) Opossum kidney cells were treated with increasing concentrations of various PPAR ligands and the potential toxic effects on cells determined by methylthiazoletetrazolium (MTT) assay. Values represent mean ± SEM, and are normalized to that observed under nonligand-treated conditions given as 100%. Each condition was performed in triplicate on three occasions. (*B*) Opossum kidney cells were treated with 5 μmol/L 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) alone (□) or together with indicated concentrations of ciglitazone (■) or troglitazone (▨). Potentially toxic effects on cells were determined by MTT assay performed in triplicate on three occasions. Values represent mean ± SEM, and are normalized to that observed in cells treated with 15d-$PGJ_2$ alone given as 100%.

All of the agonists used, except carbaprostacyclin and WY14643, demonstrated a reduction in luciferase activity at concentrations ≥10 μmol/L. Assessment of cell viability under these conditions (Fig. 3A) demonstrated that for all the agents exhibiting reduced luciferase expression impairment of cell viability developed at 10 μmol/L concentrations, very marked impairment of viability was apparent at higher concentrations. Combination of various concentrations of troglitazone with 5 μmol/L 15d-$PGJ_2$ resulted in no greater toxicity than with 15d-$PGJ_2$ alone. The addition of increasing concentrations of ciglitazone to 15d-$PGJ_2$ revealed a modest trend toward increasing toxicity. Therefore in the majority of experiments 15d-$PGJ_2$ was combined with concentrations of thiazolidinedione less than 5 μmol/L.

We next examined the phosphorylation state of PPARγ under basal conditions and after stimulation by the PPARγ agonists 15d-$PGJ_2$ and ciglitazone (Fig. 4). A representative autoradiograph showing phospho-PPARγ is depicted in Figure 4A, with a histogram representing quantification of bands in Figure 4B. Low levels of PPARγ phosphorylation were observed under control conditions. Exposure of cells to 15d-$PGJ_2$ resulted in an approximate 2.5-fold enhancement of PPARγ phosphorylation, whereas ciglitazone stimulated a lesser degree of phosphorylation. Interestingly, the combination

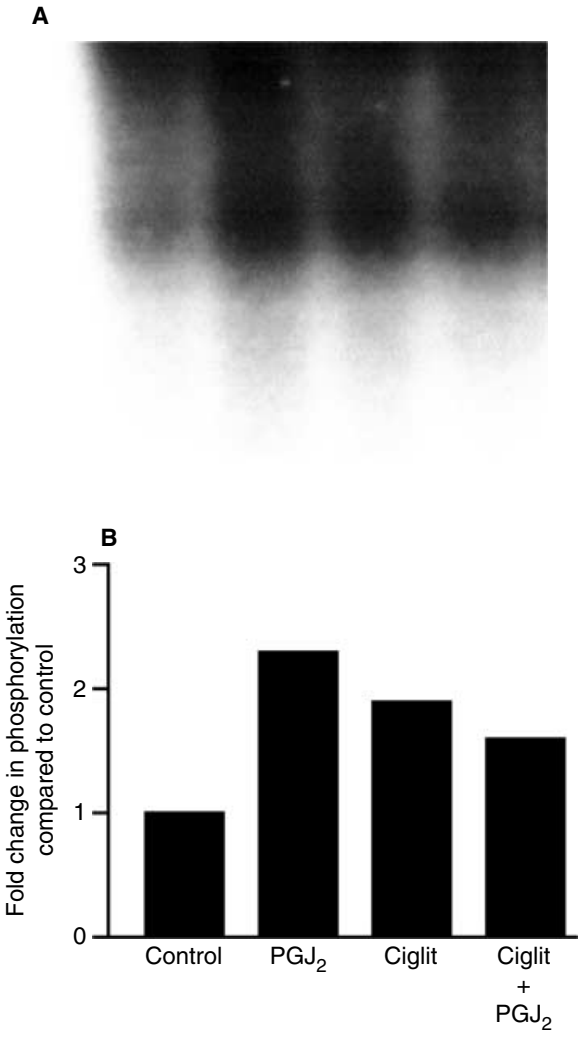


**Fig. 4. Phosphorylation of peroxisome proliferator activated receptors (PPARγ) in [$^{32}$P]-orthophosphate–labeled opossum kidney cells.** Opossum kidney cells were labeled with [$^{32}$P]-orthophosphate and stimulated with either 5 μmol/L 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$), 5μmol/L ciglitazone, or both PPARγ ligands in combination. PPARγ was then immunoprecipitated and its phosphorylation state examined by polyacrylamide gel electrophoresis (PAGE) and autoradiography. (*A*) A representative utoradiograph of phosphorylated PPARγ. (*B*) A histogram demonstrates denistometric quantification of phosphorylated bands depicted above. The level of phosphorylation observed under control, nonstimulated, noninhibitor-treated conditions has been arbitrarily assigned a value of 1. All other values have been normalized to this. The experimental condition relevant to each band of the autoradiograph is indicated below the respective histogram bar. The data depicted are representative of at least three identical experiments.

of ciglitazone and 15d-$PGJ_2$ was not associated with augmentation of the PPARγ phosphorylation observed with 15d-$PGJ_2$ alone, but rather a reduction.

To study the influence of ERK on 15d-$PGJ_2$ and ciglitazone-stimulated luciferase activity, we transfected opossum kidney cells with either pMEK1-CA or pMEK1-KD together with pPPRE-TK-luc prior to agonist stimulation (Fig. 5). In some experiments cells underwent mock transfection with irrelevant plasmid DNA as controls, and in these cells the pattern of luciferase expression in response to 15d-$PGJ_2$ and ciglitazone was as in previous experiments (Fig. 1). Transfection of cells with constitutively active MEK1 resulted in enhanced PPRE activation in unstimulated cells. Agonist treatment with either 15d-$PGJ_2$ or ciglitazone stimulated no further significant increase in PPRE activation in pMEK1-CA transfected cells. Conversely, expression of kinase dead MEK1 inhibited basal PPRE activation, and that stimulated by both 15d-$PGJ_2$ and ciglitazone.

In order to determine the ability of PPARγ agonists to stimulate ERK we used Western blotting of opossum kidney cell lysates for pERK (Fig. 6). In initial experiments we sometimes observed an early (5 minutes) apparent stimulation of ERK by 5 μmol/L ciglitazone. However, a similar response was also occasionally seen following simple media change in some experiments, and we therefore concluded that no significant activation of ERK occurred in response to this agent. No stimulation of ERK by troglitazone was observed (data not shown). In contrast, exposure of opossum kidney cells to 5 μmol/L 15d-$PGJ_2$ resulted in significant ERK activation after 30 minutes, persisting for at least 4 hours. No such pattern of activation was ever observed following media change alone, and we therefore concluded that 15d-$PGJ_2$ was able to stimulate ERK in opossum kidney cells. Very significantly both ciglitazone and troglitazone dose dependently inhibited 15d-$PGJ_2$-evoked ERK activation (Fig. 7).

Proliferation was used as an index of the functional effects of PPARγ agonists in opossum kidney cells (Fig. 8). 15d-$PGJ_2$ stimulated a robust dose-dependent increase in opossum kidney cell proliferation, whereas the thiazolidinediones ciglitazone and troglitazone had an opposite effect (Fig. 8A). When both 5 μmol/L 15d-$PGJ_2$ and varying concentrations of thiazolidinediones were applied to opossum kidney cells, a dose-dependent inhibitory effect of thiazolidinediones on 15d-$PGJ_2$-stimulated proliferation was seen (Fig. 8B).

## DISCUSSION

Various studies have examined the localization of PPAR subtypes in the kidney [5–7], but their physiologic functions in this organ and potential roles in kidney disease are not well understood. For PPARγ both protective and detrimental influences on kidney cell behavior have been described [8–13]. For instance, we demonstrated in primary human proximal tubular cells that activation of PPARγ by albumin-bound fatty acids and 15d-$PGJ_2$ resulted in apoptosis [13]. We postulated that this effect might be responsible in part for tubular atrophy observed in proteinuric renal disease. Additionally, we suggested that other PPARγ ligands could possess the potential to prevent PPARγ-mediated fatty acid–induced proximal

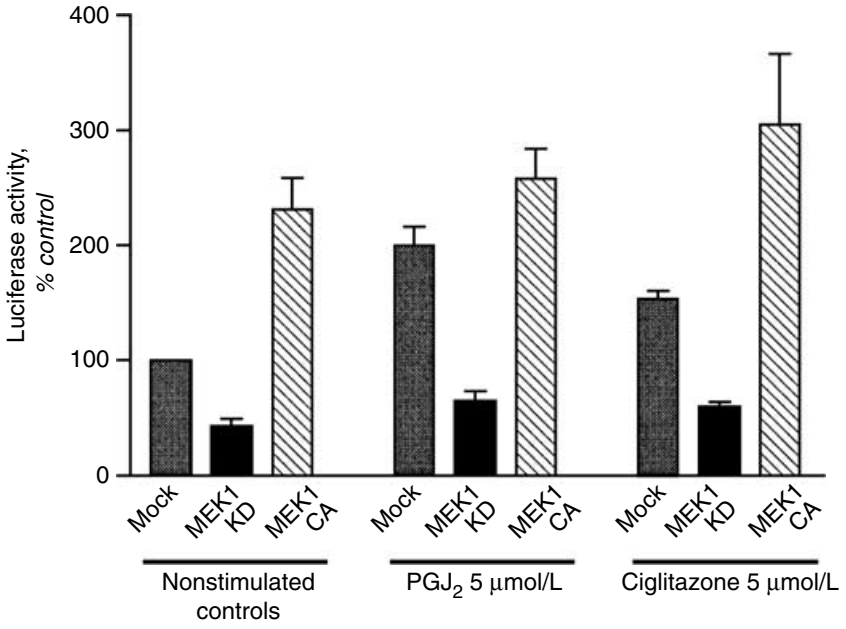


**Fig. 5. Activation of peroxisome proliferator activated receptors (PPAR) response elements (PPRE) by PPARγ ligands in cells transfected with kinase dead or constitutively active mitogen-activated protein (MAP) kinase kinase (MEK1)**. Opossum kidney cells were transiently transfected with pPPRE-TK-luc together with either pMEK1-CA or pMEK1-KD, or mock transfected with irrelevant DNA. Luciferase activity was measured in non-stimulated cells or cells stimulated with the indicated PPARγ ligands. Luciferase values are given as a percentage of that seen in mock transfected, nonstimulated controls. Values are expressed as means ± SEM. Each condition was measured in triplicate in four experiments.

tubular toxicity [13]. Despite the previous observations that PPARα was the most highly expressed subtype in the kidney proximal tubule [5–7] we demonstrated that PPARγ agonists rather than PPARα agonists elicited the greatest activation of PPRE in human proximal tubular cells [13]. We now report similar findings in opossum kidney proximal tubular cells. In the current study only PPARγ ligands stimulate appreciable PPRE activity in opossum kidney cells. In fact, PPARα ligands fail to activate PPRE, and PPARβ ligand-stimulated PPRE activity is trivial. With regard to PPARγ ligands it is significant that 15d-$PGJ_2$ exhibited a more potent PPRE stimulatory effect than two different thiazolidinediones, ciglitazone and troglitazone, in all our experiments, such that despite increasing concentrations thiazolidinediones only partially activate PPRE.

We went on to examine the differential effects of thiazolidinediones and 15d-$PGJ_2$ on signaling pathways, and cell functional events known to be associated with PPARγ mediated activities. Despite both being high-affinity ligands for, and agonists of, PPARγ [33, 34], important differences between the activities of 15d-$PGJ_2$ and thiazolidinediones in opossum kidney cells are revealed in this study. Whereas 15d-$PGJ_2$ and 9-cis retinoic acid exert a synergistic effect on PPRE activation, no such synergy is observed between 9-cis retinoic and ciglitazone. Both ciglitazone and troglitazone reverse 15d-$PGJ_2$–induced PPRE activation, resulting in levels of PPRE activation similar to those observed with the thiazolidinedione alone. Ciglitazone induces less phosphorylation of PPARγ than 15d-$PGJ_2$, and inhibits 15d-$PGJ_2$–stimulated phosphorylation PPARγ. Activation of ERK by 15d-$PGJ_2$ is also inhibited by both thiazolidinediones, as is 15d-$PGJ_2$–stimulated proliferation of opossum kidney cells, although ciglitazone displays the greatest potency in this respect.

Other interesting points emerge from the current study in relation to the stimulation of ERK by PPARγ ligands and its role in activation of PPARγ. In many published studies activation of ERK, and ERK-mediated phosphorylation of PPARγ is associated with a reduction in its transcriptional activity [24, 26–28, 35, 36]. However, the data from the current experiments using mutant MEK1, performed in a physiologically relevant cell line expressing only endogenous PPARγ rather than overexpressed recombinant PPARγ, clearly establish that in opossum kidney cells ERK activity is a prerequisite for efficient stimulation of PPRE, and that ERK inhibition abolishes PPRE activation both under basal conditions and when stimulated by PPARγ ligands. Nonetheless, in opossum kidney cells endogenous PPARγ is clearly phosphorylated in response to ligand activation, more so by 15d-$PGJ_2$ than by ciglitazone. The 15d-$PGJ_2$–mediated stimulation of PPARγ phosphorylation may be mediated by ERK because this agent itself stimulates ERK activity. It seems unlikely that the increased PPARγ phosphorylation evoked by ciglitazone is ERK mediated, however, because ERK is not stimulated under this condition. Other kinases such as c-Jun N-terminal kinase have been shown to phosphorylate PPARγ [37] but further identification of the kinase responsible is beyond the scope of this study.

According to the system under study 15d-$PGJ_2$ and thiazolidinediones have been shown to have varying effects on ERK activity. In certain studies both PPARγ ligands stimulate ERK, whereas in some only one or the other

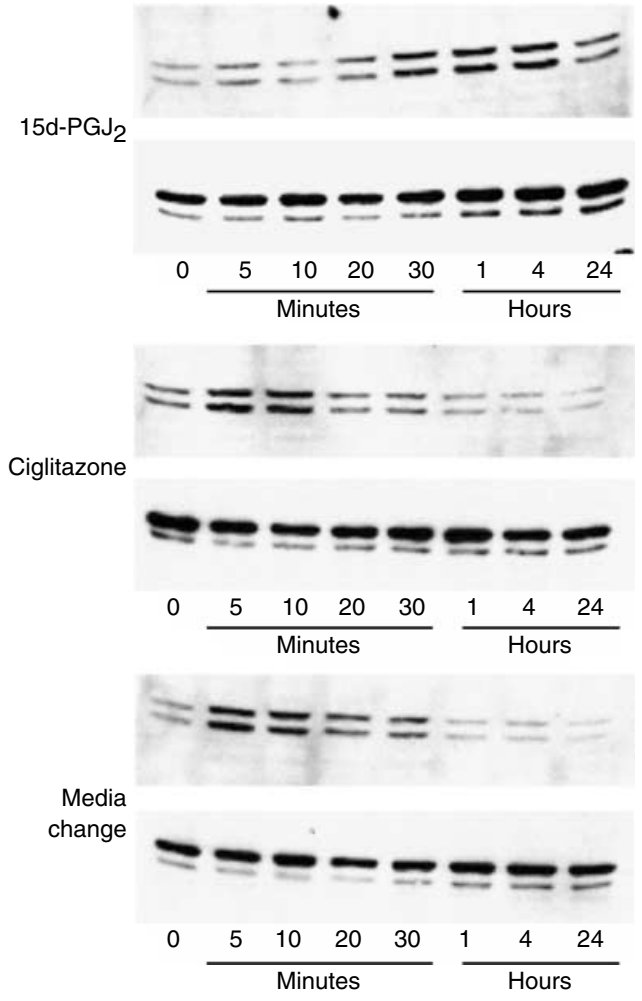


**Fig. 6. 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) but not ciglitazone activates extracellular signal-regulated protein kinase (ERK) in opossum kidney cells.** Opossum kidney cells were exposed to 5 μmol/L 15d-$PGJ_2$, 5 μmol/L ciglitazone or media change and lysed after various times. Lysates were subjected to immunoblotting for either pERK (upper panel for each condition) or total ERK (lower panel for each condition). Immunoblots shown are representative of at least three experiments.

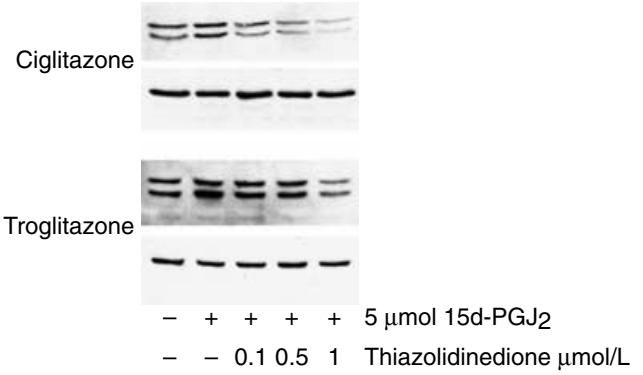


**Fig. 7. Thiazolidinediones inhibit 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) stimulated extracellular signal-regulated protein kinase (ERK) activity in opossum kidney cells.** Lysates from opossum kidney cells stimulated with both 15d-$PGJ_2$ and increasing concentrations of ciglitazone (upper pair of panels) or troglitazone (lower pair of panels) were immunoblotted for pERK (top panel of each pair) or total ERK (lower panel of each pair). Immunoblots are representative of three identical experiments.

has this ability [29, 31, 38–40]. Emerging evidence implicates PPARγ-independent mechanisms in the activation of ERK by these agents. However, in our study only 15d-$PGJ_2$ was able to activate ERK and for two reasons the results suggest, although do not definitively prove, that activation of ERK by 15d-$PGJ_2$ is mediated directly via PPARγ. First, the activation kinetic for ERK by 15d-$PGJ_2$ is relatively long and sustained, thus being compatible with a transcription dependent event. Second, it is antagonized by ciglitazone and troglitazone, two different PPARγ ligands.

A variety of effects of different PPARγ ligands on cell growth and proliferation have been described depending on the ligand used and/or the cell type studied [41–43]. Such observations have stimulated considerable interest in the roles of PPAR ligands in cancer and inflammation, although few studies in kidney cells have been performed. In our previous studies of human proximal tubular cells both 15d-$PGJ_2$ and fatty acid PPARγ ligands stimulated apoptosis of kidney cells, as did overexpression of PPARγ [13]. However, kidney cell responses to 15d-$PGJ_2$ appear to be context specific, varying with applied concentration and cell type studied. Rovin et al [44] found that at low concentrations 15d-$PGJ_2$ stimulated mesangial cell proliferation, but at higher concentrations induced cell death. Thiazolidinediones at low concentrations were less potent stimulators of proliferation than 15d-$PGJ_2$ but at higher concentrations the ability of these agents to induce apoptosis was similar. The results of our studies are similar in that 15d-$PGJ_2$ stimulated robust proliferation of proximal tubular cells at low concentration, whereas thiazolidinediones had an inhibitory effect on cell proliferation. Furthermore, both ciglitazone and troglitazone at low concentration were able to antagonize the proproliferative effect of 15d-$PGJ_2$. Our toxicity data suggest that cell death would likely occur at higher doses of these compounds, and that any proliferative responses would be abolished. However, it was not our aim to study this directly.

The current findings that thiazolidiendiones (*1*) fail to elicit full PPRE activation; (*2*) inhibit 15d-$PGJ_2$–mediated PPRE activation; (*3*) block 15d-$PGJ_2$–induced PPARγ phosphorylation; (*4*) prevent 15d-$PGJ_2$ stimulation of ERK; and (*5*) stop 15d-$PGJ_2$–mediated proliferation indicate that in opossum kidney cells two different thiazolidiendiones behave as partial PPARγ agonists compared to 15d-$PGJ_2$. Other PPARγ-binding thiazolidinedione and nonthiazolidinedione compounds have

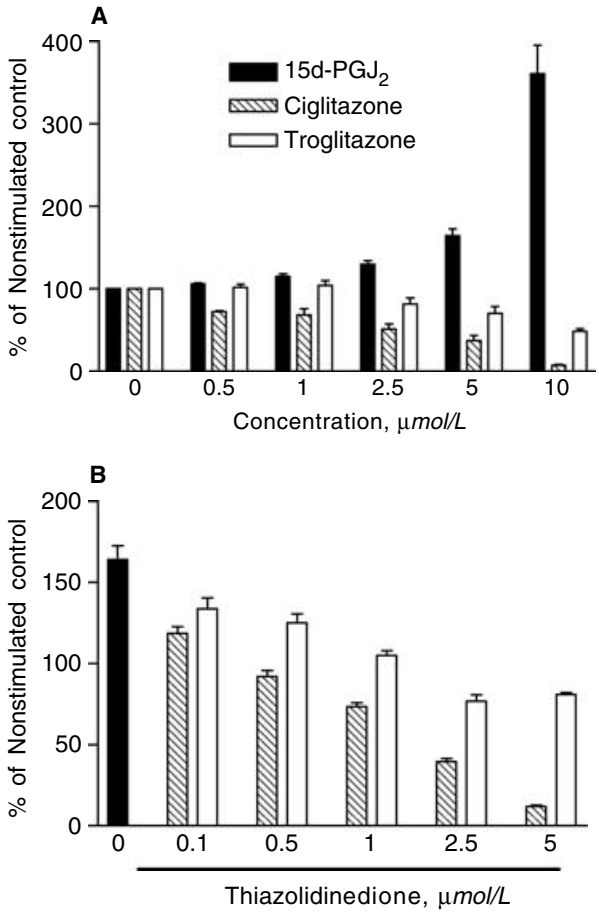


**Fig. 8. Proliferative responses of opossum kidney cells exposed to 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) and thiazolidinediones.** (*A*) Opossum kidney cells were exposed to increasing concentrations of either 15d-$PGJ_2$, ciglitazone, or troglitazone. Proliferation was measured by [$^3$H] thymidine incorporation and counts accumulating in nonstimulated cells were arbitrarily assigned a value of 100%. (*B*) Opossum kidney cells were stimulated with 5 μmol/L 15d-$PGJ_2$ alone (■) or 5 μmol/L 15d-$PGJ_2$ together with increasing concentrations of ciglitazone (□) or troglitazone (□). Proliferation was measured by [$^3$H] thymidine incorporation and values are expressed as a percentage of those obtained from nonstimulated cells. Values are expressed as means ± SEM. Each condition was measured in triplicate in four experiments.

been shown to have partial agonist activity. Reginato et al [45] described MCC-555, a thiazolidinedione with context specific effects on PPARγ transcriptional activity. Depending on the cell type studied this agent was able to act as a full agonist, partial agonist, or antagonist. Another compound, GW0072, binds PPARγ with high affinity but exhibits weak partial agonist activity with a relative efficacy equal to ~20% of rosiglitazone, while antagonizing the effects of rosiglitazone itself [46]. Rosiglitazone activation of PPRE can also be blocked by the nTZpa, a nonthiazolidinedione PPARγ ligand [47]. Most recently Misra et al [48] described a PPARγ ligand thiazolidinedione, PAT5A, which interacts differently with PPARγ leading to differential cofactor recruitment and thus gene activation compared to rosiglitazone.

To the best of the authors' knowledge this is the first study to demonstrate partial agonist effects of established thiazolidinediones. The results of the current study indicate a pharmacologic basis for the use of thiazolidinediones to combat progressive proteinuric renal disease, such that a partial agonist thiazolidinedione may be employed to inhibit detrimental effects of other PPARγ ligands in proximal tubule and other kidney cells.

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*Reprint requests to Dr. Nigel J. Brunskill, Department of Cell Physiology and Pharmacology, Faculty of Medicine and Biological Sciences, Medical Sciences Building, University Road, Leicester LE1 9HN England.*
*E-mail: njb18@le.ac.uk*

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